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Three Loci Modify Growth of a Transgene-Induced Mammary Tumor: Suppression of Proliferation Associated with Decreased Microvessel Density

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In earlier studies it was observed that the genetic background significantly affected the phenotype of a transgene-induced mammary tumor. Tumors arising in an (I/LnJ × PyMT) F_1 hybrid background appeared earlier than in the FVB/N-TgN(MMTV-PyVT)^{634Mul} parent, but accumulated less tumor mass, indicating a net decrease in tumor growth. Quantitative genetic mapping in a backcross identified three loci that were associated with the decreased proliferative capacity of the I/LnJ F_1 tumors. Molecular analysis of the tumors suggests that these loci may act by restricting the tumor's ability to recruit microvessels. The three loci, designated *Mmtg1–3,* are unlinked to the angiogenic genes *Fgf2, Flt1, Flk4, Flk1, Vegf,* and *Vegfc,* as well as the precursors of the endogenous antiangiogenic molecules angiostatin and endostatin. The *Mmtg* loci may therefore provide novel targets for antiangiogenic therapeutic strategies.

INTRODUCTION

The clinical importance of breast tumor growth rate has been a subject of many investigations. A wide range in tumor doubling rates has been reported in the literature (Spratt and Spratt, 1976). More rapidly growing tumors usually have poorer outcomes, which has led to tumor doubling time being used as a prognostic factor (Collins, 1968; Kusama *et al.,* 1972). More recently molecular markers of tumor proliferation have been used to determine the correlation between breast cancer survival and tumor proliferation. Significant associations linking high proliferative indexes with patient mortality were observed with *in vivo* labeling with BrdU (Gaglia *et al.,* 1993; Meyer *et al.,* 1989, 1993) or [^{3}H]thymidine (Meyer and Province, 1988), flow cytometry measurements, and immunohistochemical staining with the proliferation markers Ki-67 and PCNA (Gaglia *et al.,* 1993; He *et al.,* 1994; Magno *et al.,* 1992). These results suggest that a comprehensive understanding of all of the factors that influence tumor proliferation and their correlation with long-term outcome would be potentially of great clinical benefit. It may be possible to design therapeutic strategies to specifically target critical factors in tumor proliferation and thereby extend the long-term survival rate of patients, particularly those with highly proliferative tumors, who have the poorest outcomes (Aaltomaa *et al.,* 1992a,b).

A major determinant of tumor growth rate is the balance between cellular proliferation and apoptosis. Both intrinsic and environmental factors can play major roles in these processes. For example, defects in cell cycle regulatory checkpoints or activation of proliferative signaling pathways would be expected to play a major role in determining the rate of tumor expansion. Growth-stimulatory signals can also be counterbalanced by increased sensitivity to apoptosis due to genomic damage or mutations, resulting in a lower net growth rate than would be predicted by proliferative index. In addition to these intrinsic factors, environmental factors also influence the ability of a tumor to grow. A major subject of recent interest is tumor angiogenesis. The ability of the tumor to recruit a high density of microvesicles is critical to provide an adequate supply of nutrients to enable growth (Belien *et al.,* 1999; Wu, 1996). Furthermore, exposure to soluble growth-stimulatory molecules, determined both by the concentration present in the host and by accessibility to the tumor, might also be expected to have a major impact on tumor proliferation.

A further level of complexity is added to the intricate biochemical interactions at the genetic level. Allelic variations that alter enzyme kinetics or intermolecular interactions may also alter net tumor growth by subtly changing one or more metabolic pathways (Matsui *et al.,* 2000; Zunarelli *et al.,* 2000). These genetic polymorphisms, rather than loss or gain of function, are likely to be responsible for the large number of strain-

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specific variations in phenotype observed in inbred mouse strains for a variety of traits, including tumor susceptibility, alcohol and narcotic preference, and diabetes (Frankel, 1995). Identification of polymorphic genes that affect tumor biology in inbred mouse strains and characterization of how variants affect tumor phenotype may provide valuable clues for tumor biology that can be applied in a heterogeneous human clinical setting.

Previously we described the analysis of a backcross between FVB/N-TgN(MMTV-PyVT)^{634Mul} (Guy *et al.*, 1992) and I/LnJ to map genes associated with acceleration of a transgene-induced mammary tumor (Le Voyer *et al.*, 2000). We demonstrated that there was no difference in expression levels of the transgene in the two genetic backgrounds and the change in latency was due to an epistatic interaction between genes on chromosomes 9 and 15. During this analysis it was observed that the kinetics of tumor growth was also significantly altered by the introduction of the I/LnJ genome. Accumulation of tumor tissue in the [I/LnJ × FVB/N-TgN(MMTV-PyVT)^{634Mul}] F_1 animals was reduced approximately 20% ($P = 0.009$) compared to the FVB/NJ parent, suggesting the presence of modifiers of tumor growth kinetics as well as latency in the I/LnJ genome. In this study we report the identification of three mammary tumor growth modifier loci, two of which map to regions independent of the latency modifier loci. Furthermore, molecular analysis suggests that the modifiers possibly affect the ability of tumors to recruit internal microvessels. Identification and characterization of these tumor growth modifiers may provide important insights into mammary tumor biology and potential utility in the clinical setting for diagnostic or therapeutic intervention.

MATERIALS AND METHODS

Animals. FVB/N-TgN(MMTVPyVT)^{634Mul} mice were obtained from W. Muller, McMaster University, Hamilton, Ontario, Canada. FVB/NJ and I/LnJ mice were purchased from The Jackson Laboratory. The generation and genotyping of the backcross was described in Le Voyer *et al.* (2000).

Determination of tumor burden. Primary mammary tumors were diagnosed by palpation on alternate days. Animals were palpated three times a week to detect the primary mammary tumor. At diagnosis the animals were weighed and then aged for an additional 40 days. At sacrifice, the carcass was weighed and the difference in the weights used as an approximate measure of total tumor burden. The mammary tumors of a subset of animals ($N = 6$) were dissected and weighed to confirm that the approximate tumor burden determined by the change in weight was consistent with direct measurement of the tumor mass. On average greater than 80% of the change in weight was due to accumulation of tumor tissue (70–90%). The F_1 and N_2 animals are slightly smaller than the FVB/NJ parents. The average body weights at diagnosis were 21.3 g for FVB ($n = 78$), 18.2 g for the (I/LnJ × PyMT) F_1's ($n = 36$), and 19.6 g for the N_2 animals ($n = 126$). However, since the F_1 animals' average tumor latency was 38 days after birth and the N_2's latency 46 days after birth compared to 56 days for the homozygous FVB, some of the tumor burden measured is due to normal body growth (15–20% of the tumor mass). Therefore the tumor burden for the F_1's and N_2's is a conservative overestimate of their actual tumor mass.

Immunohistochemistry. Nonnecrotic tumors were isolated 40 days after detection. Tissues were fixed in neutral-buffered formalin and then paraffin embedded and sectioned. Immunohistochemical stains were performed with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), following the manufacturer's protocol. Immunohistochemistry was performed by the Fox Chase Cancer Center Experimental Histopathology Center.

Microvessel density measurement. Nonnecrotic tumors were used for the analysis. Histological examination of the FVB/NJ and I/LnJ F1 tumors demonstrated no differences in pathological type. Tumors were selected blindly from the collection to prevent investigator-induced bias. Three tumors of each genotype were examined. Microvessel density was measured using a Leica Q500IW image analysis workstation. Images were captured using a Leica M420 microscope at 32× magnification. Area of tumor tissue in each field as well as the number of microvessels was automatically measured using a custom computer macro. Three fields per slide were analyzed.

Apoptosis assays. Apoptosis assays were performed with the ApoTag Plus Peroxidase *in situ* detection kit (Intergen, Purchase, NY) according to the manufacturer's recommended protocol. Apoptotic index was determined with a Leica Q500IW imaging work station by measuring the number of TUNEL-positive cells per field of view.

Western blots. Tumors were homogenized in lysis buffer (1× PBS, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.004% NaF, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM Na orthovanadate, pH 7.4) and centrifuged at 100,000*g* to pellet insoluble material. The protein concentration of the supernatant was determined by the BCA method (Bio-Rad). Western blots were performed basically as described (Ausubel *et al.*, 1997). Briefly, 10 μg of protein from each sample was electrophoresed on a 10% SDS–PAGE gel and electroblotted onto an Optitran membrane (Schleicher & Schuell). The filter was blocked in 5% nonfat dry milk in PBS^- for 1 h, washed, and incubated with the primary antibody in 1% nonfat dry milk in PBS^-. The filter was washed and incubated with the secondary HRP-conjugated antibody. The ECL detection system (Amersham) was used for detection, and the filter was exposed on Hyperfilm for 5 min. The manufacturer's recommended dilution of the primary antibody was used for all analyses.

QTL analysis. The tumor burden data were transformed to fit a normal distribution and then analyzed with the QTL mapping program Map Manager QT. Sixty-nine loci (average spacing ~20 cM) were used for haplotype analysis of the 126 animals in the cross (Le Voyer *et al.*, 2000). The dataset was mapped using Map Manager QT (Manly and Olson, 1999). The backcross tumor burden data were log transformed to approximate a normal distribution. Appropriate statistical thresholds for mapping QTLs (Lander and Kruglyak, 1995) were estimated by permuting the correctly ordered dataset 10,000 times using the Doerge and Churchill (1996) algorithm implemented by Map Manager. Each permutation was mapped at 1-cM intervals across the entire genome. Statistical analysis was performed using the Statistica Package (Statsoft, Tulsa, OK).

RESULTS

Genetic background influences tumor growth. During the initial strain survey (Lifsted *et al.*, 1998) performed to identify modifiers of the transgene-induced mammary tumors it was observed that the contribution of the I/LnJ genetic background had a significant effect on final tumor burden (see Fig. 1). Comparison of the tumor burden between FVB/N-TgN(MMTV-PyVT)^{634Mul} and (I/LnJ × PyMT) F_1 animals by Student's *t* test demonstrated an ~20% reduction in final tumor burden in the I/LnF_1 animals ($P = 0.009$). To determine whether the difference in tumor growth rate might be related to the difference in latency in the two

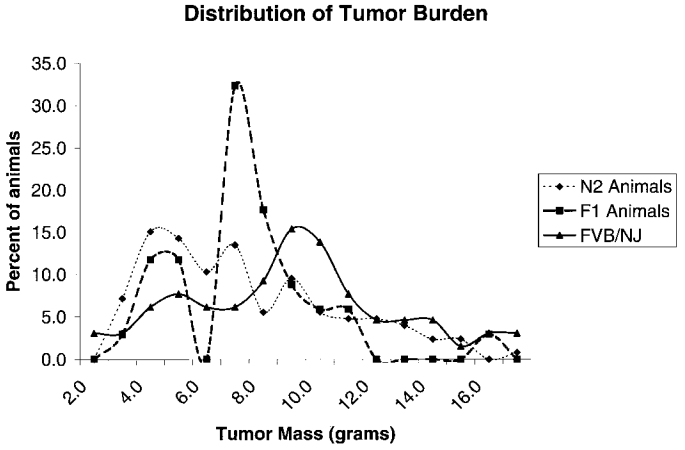

FIG. 1. Tumor burden distribution of FVB/NJ-TgN(MMTV-PyMT)^{634Mul}, (I/LnJ × PyMT) F_1, and (I/LnJ × FVB/NJ) × FVB/NJ N_2 animals. The *x* axis represents the approximate tumor mass as represented by the change in weight of the animals between diagnosis of the primary tumor and sacrifice. The *y* axis represents the percentage of animals in each class in each tumor burden range.

genotypes, the backcross was examined to see if there was any correlation of latency with tumor burden. Spearman rank order correlation was performed and no significant association was observed ($N = 126$, $R = 0.07$, $P = 0.44$). In addition, the number of tumors observed as well as the location of the tumors was also examined. No differences were observed for either tumor number or position of the tumors. These results suggested that there were additional modifier genes present in the I/LnJ inbred background, independent of the latency-modifying genes. The backcross was therefore reanalyzed with the tumor burden data to identify the putative location of these modifier genes.

QT mapping. Three peaks were detected using the Macintosh program MapManager QT that met or exceeded the recommended LOD 3.3 threshold for significance in a genome-wide scan (Fig. 2). One peak was observed on the proximal third of chromosome 7, probably close to the centromere. Two separate peaks were observed on chromosome 4, approximately 31 and 56 cM distal to the centromere. The chromosome 7 locus accounted for 6.8% of the variance and the proximal and distal chromosome 4 loci account for 3.0 and 9.4%, respectively. Previous analysis of this backcross demonstrated epistatic interaction between loci that modified tumor latency. ANOVA of these three loci was therefore performed to determine whether tumor burden might also have an epistatic component. No evidence of an interaction was observed for any of the locus combinations ($P > 0.27$), suggesting that these loci act additively, rather than interactively. The proximal and distal chromosome 4 loci were designated *Mmtg1* and *Mmtg2*, respectively (Modifier of mammary tumor growth), and the locus on chromosome 7 as *Mmtg3*.

Proliferation and apoptosis analysis. The difference in accumulation of tumor mass could be explained by either different rates of tumor apoptosis in the two genotypes or different rates of tumor proliferation or a combination of the apoptosis and proliferative differences. TUNEL assays were performed on paraffin-embedded sections to determine whether there was a significant difference in the degree of apoptosis in the FVB/NJ ($N = 3$) or (I/LnJ × PyMT) F_1 tumors ($N = 3$). No differences were observed (Fig. 3). Western blots were performed to determine whether there was a significant difference in the number of cycling cells in the different tumors. As can be seen in Fig. 4, the FVB/NJ tumors had a greater amount of Ki-67 antigen present. These results suggest that the difference in tumor accumulation observed between the FVB/NJ and the (I/LnJ × PyMT) F_1 tumors was due to different growth rates of the tumor cells, rather than different balances between proliferating and apoptotic cells.

Angiogenesis analysis. Possible explanations for difference in proliferation of the FVB/NJ and (I/LnJ × PyMT) F_1 tumors would be an inherently different tumor growth rate due to differences in cell cycle regulation or variations in tumor microenvironment, for example, access to nutrients due to differences in tumor vascularization. To address this question, paraffin sections of FVB/NJ and (I/LnJ × PyMT) F_1 tumors were stained for Cd31, a vascular endothelial marker, and the density of microvessels quantitated using a Leica Q500IW Image Analysis workstation and compared by the Mann–Whitney *U* test (Fig. 5). No significant difference in microvessel density was observed on the periphery of the tumor ($P = 0.4$). However, a significant difference was observed on the interior of the tumor ($P = 0.002$) with the FVB/NJ tumors having an approximately fivefold greater density of microvessels compared to the (I/LnJ × PyMT) F_1 tumors (84.2 vs 14.0 Cd31-positive staining foci per 10^6 μm^2 of tumor, range 54.6–126.7 for FVB/NJ and 10.0–21.7 for (I/LnJ × PyMT) F_1) (Fig. 6).

DISCUSSION

Previously our laboratory demonstrated the presence of genes in the I/LnJ inbred mouse strain that modify the tumor latency of a polyoma middle T-induced mammary tumor. Two interacting genes were present on chromosomes 9 and 15, and a third potential gene mapped to proximal chromosome 7. In this study we have demonstrated the presence of three modifier loci that significantly affect the growth rate of the transgene-induced mammary tumor. Two of these growth-modifying loci (*Mmtg1* and *Mmtg2*) map to chromosome 4 and therefore are novel modifier loci. The third locus, *Mmtg3*, maps to proximal chromosome 7, the same region where the third potential latency modifier gene was localized. Although it cannot definitively be ruled out that these loci are identical, we believe that they are probably distinct loci because there was no evidence of a correlation between tumor latency and tumor growth as might be expected if the

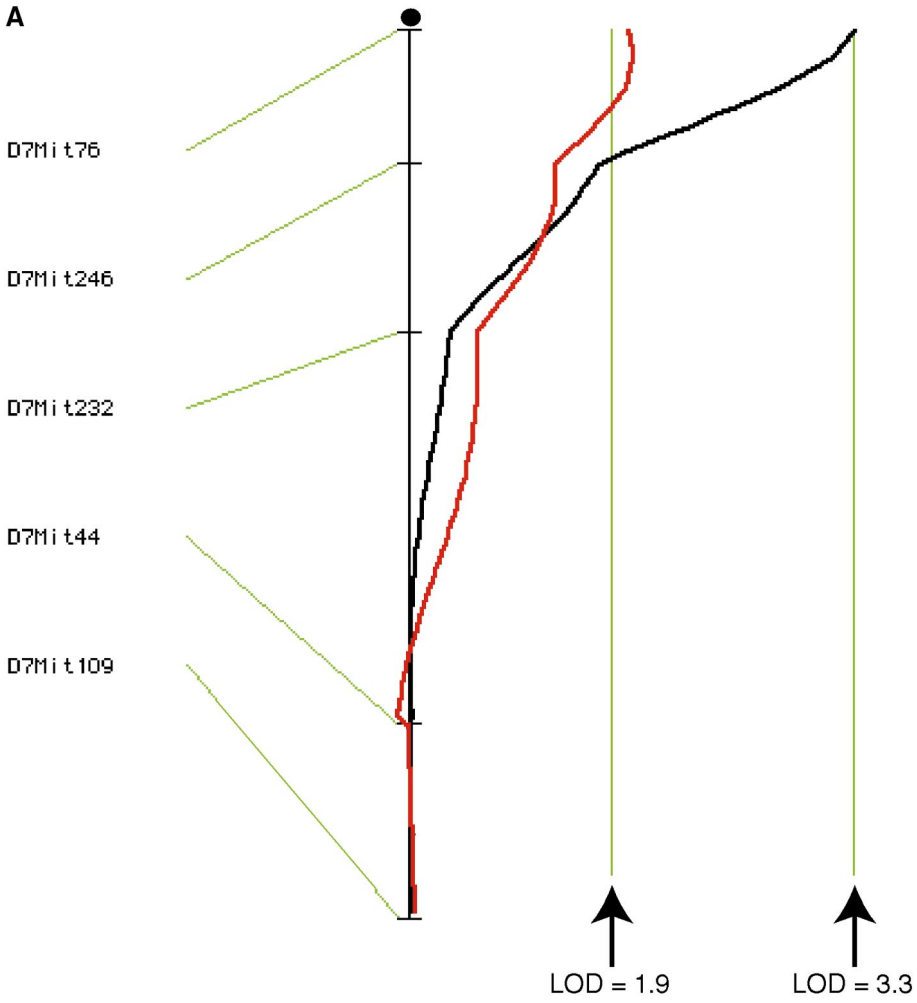

FIG. 2. Schematic representation of the quantitative trait mapping results. The maps are oriented with the centromere at the top and the loci used in the segregation analysis shown to the left. The black line to the right of the chromosome indicates the probability of a gene associated with the change in tumor burden being present at that position. The suggestive and significant lod scores of 1.9 and 3.3 are indicated with the vertical green lines. The red line represents the additive effect of the loci, positive if it is to the right of the chromosome, negative if it is to the left. (**A**) Chromosome 7; (**B**) chromosome 4.

same gene affected both traits. In addition, the mapping data suggest that the QTL mapping peaks are likely to be distinct, with the apex of the *Mmtg3* peak being close to the centromere and the latency peak somewhat more distal. However, due to the close proximity of the peaks and the lack of resolution of quantitative trait mapping, it is certainly conceivable that the loci are one and the same.

Mmtg1 and *Mmtg2* map to regions of mouse chromosome 4 that have been implicated in mammary tumorigenesis in a number of studies (Cool and Jolicoeur, 1999; Radany *et al.*, 1997; Ritland *et al.*, 1997; Weaver *et al.*, 1999). Loss of heterozygosity on chromosome 4 has been reported for three different transgenic models, including distinct regions of loss encompassing both the *Mmtg1* and the *Mmtg2* regions (Cool and Jolicoeur, 1999; Radany *et al.*, 1997; Ritland *et al.*, 1997; Weaver *et al.*, 1999). Loss of heterozygosity is not observed in the polyoma transgene model used in this study (Ritland *et al.*, 1997), although like the MMTV-*myc* transgenic model specific translocations have been observed between chromosomes 4 and 11 (Weaver *et al.*, 1999; K.H., unpublished observations). Finally, loss of heterozygosity has been observed in chemically induced mammary tumors, with a high degree of loss observed in advanced, transplantable tumors (Aldaz *et al.*, 1996). These results have been interpreted to suggest the presence of at least two tumor suppressor genes on chromosome 4. This interpretation is supported by the fact that mouse chromosome 4 is homologous with human chromosomes 1p and 9p (Blake *et al.*, 2000), which have also been associated with loss of heterozygosity in human breast cancer (Bieche and Lidereau, 1995; Devilee and Cornelisse, 1994; Driouch *et al.*, 1998).

Although *Mtmg1* and *Mtmg2* map to these regions of common LOH in mammary tumors, it is unclear whether the putative tumor suppressors and the tumor growth modifiers described here are one and the same. Experiments in our laboratory and others have demonstrated a lack of LOH in the polyoma middle-T-induced mammary tumors on these chromosomes, ar-

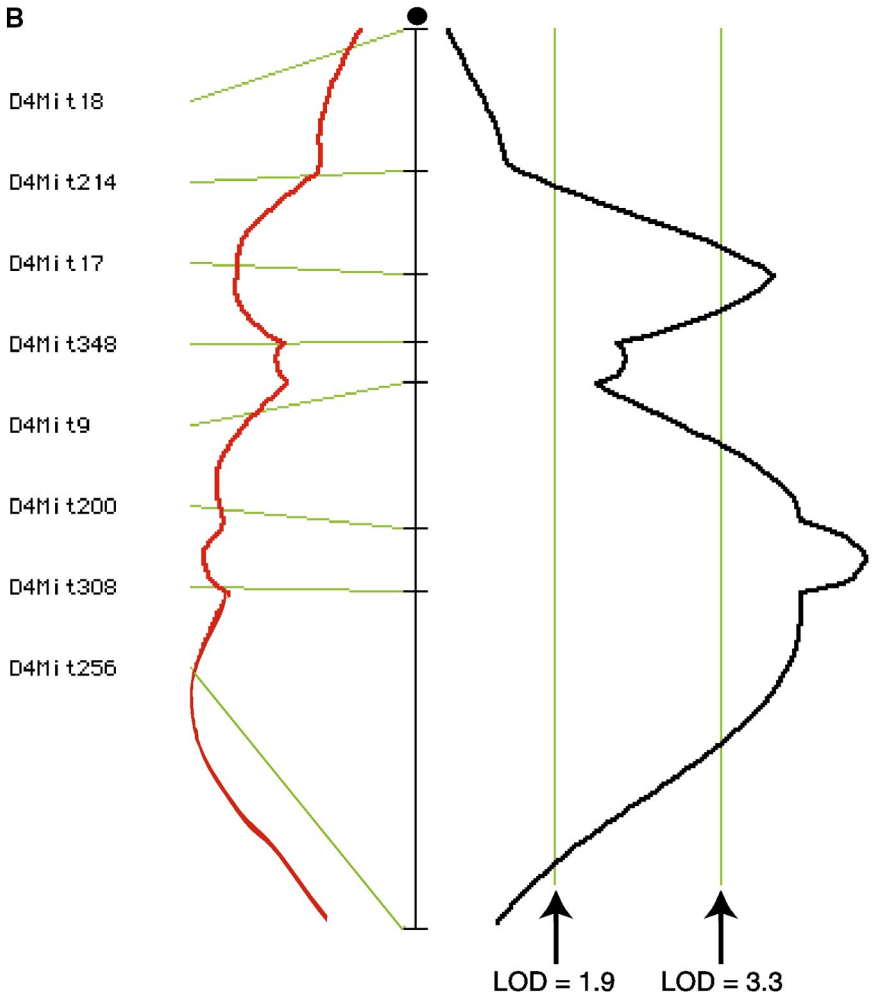

FIG. 2—*Continued*

guing against a role for a traditional tumor suppressor gene in this model (Ritland *et al.*, 1997; K.H., unpublished results). Nor is it clear that the *Mtmg* loci function exclusively in mammary tumors. It is possible that these loci may have a growth-suppressive effect on multiple tumor types and by coincidence map to Chr 4, much like the plasmacytoma modifiers *Pctr1* and *Pctr2* (Potter *et al.*, 1994) and the Apc^{min} modifier *Mom1* (Gould *et al.*, 1996). Because of the lack of LOH associated with this tumor model, we currently favor the hypothesis that the *Mtmg* loci and the tumor suppressors are distinct. The resolution of this question, however, will have to wait until all of the genes are identified.

The most likely causes of different tumor growth rates in the two genetic backgrounds are different rates of progression through the cell cycle, different proportion of cells undergoing apoptosis, or unequal access to nutrients and growth factors due to differences in tumor vasculature. To assess these possibilities we performed a combination of Western blotting and immunohistochemistry analysis. Ki-67 Western blots demonstrated that the (I/LnJ × PyMT) F_1 tumors contained lower levels of the proliferation-specific antigen than the FVB/NJ tumors. Ki-67, an antigen specifically expressed in dividing cells, has been positively correlated with mitotic index. This result therefore indicates that fewer cells were progressing through the cell cycle in the (I/LnJ × PyMT) F_1 tumors compared to the FVB/NJ inbred background. Examination of apoptosis within the tumors, which arose in the two genotypes, did not reveal any significant differences, leading to the conclusion that the difference in growth rate was due to fewer cells undergoing division in the (I/LnJ × PyMT) F_1 tumors rather than changes in the proliferative–apoptotic balance).

There are a number of reasons why the mitotic index might be different between the two genetic backgrounds. One explanation might be that either the (I/LnJ × PyMT) F_1 tumor cells have inherent lower proliferative capacity than FVB/NJ cells. Alternatively, FVB/NJ tumors might have greater access to nutrients and growth factors compared the (I/LnJ × PyMT) F_1 tumors. Immunohistochemistry was performed to assess directly the degree of tumor vascularization. No difference was observed in the microvessel density on the capsule and surrounding stroma. Unexpectedly, a significant difference was observed in the

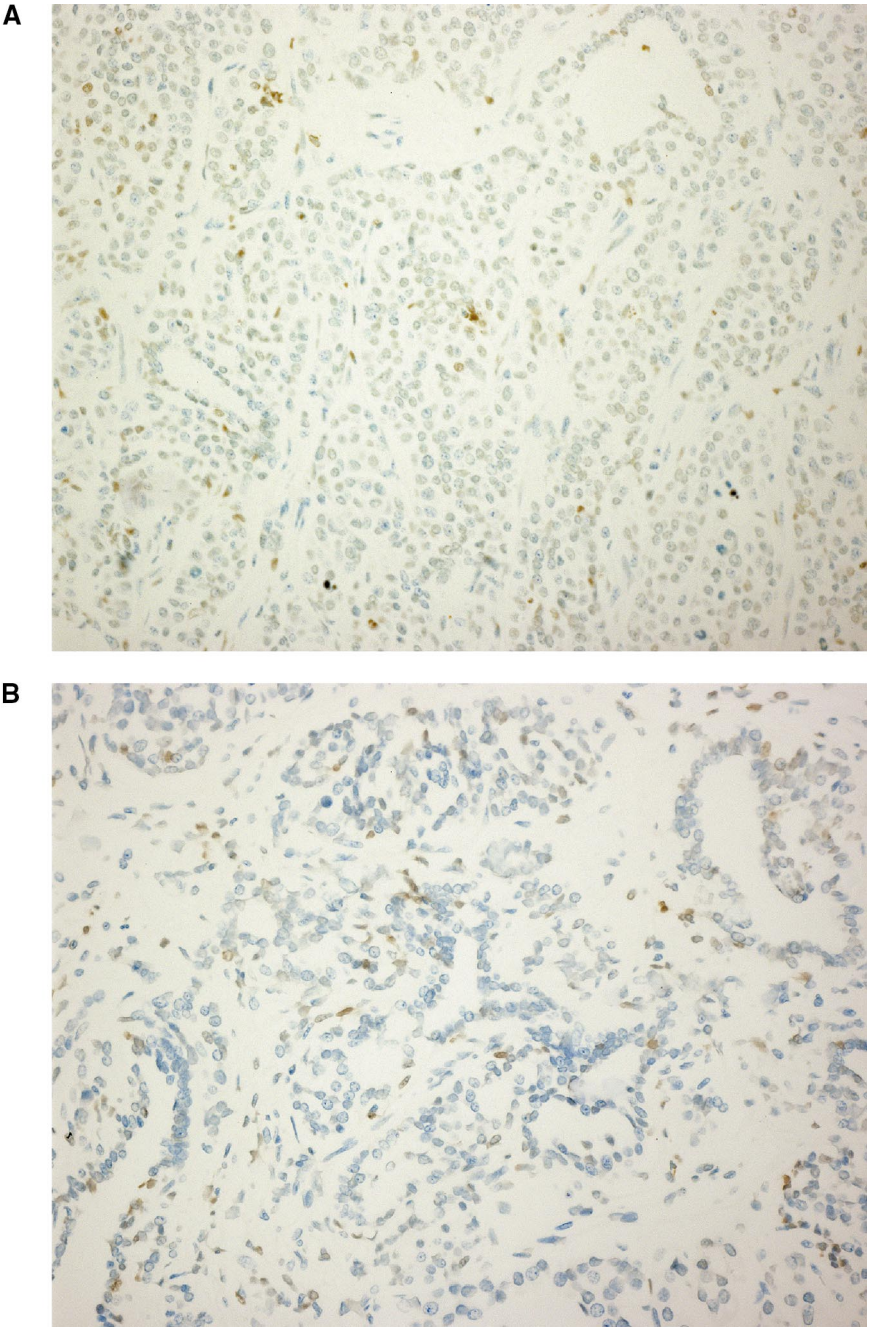

FIG. 3. TUNEL assay of tumors to compare rates of apoptotic cells. (**A**) FVB/NJ tumor; (**B**) (I/LnJ × PyMT) F_1 tumor.

microvessel density in the interior of the tumor. FVB/NJ tumors had on average a fivefold greater density of intratumor microvessels than the slower growing (I/LnJ × PyMT) F_1 tumors (see Fig. 5). The difference in growth rates is therefore likely to be due simply to greater accessibility to oxygen, growth factors, and other nutrients, although at this time we cannot rule out other contributing mechanisms.

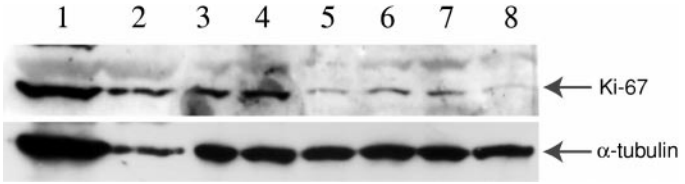

FIG. 4. Ki-67 Western blot. Lanes **1,** transgene-negative FVB/NJ 30-day-old mammary gland; **2,** transgene-negative 30-day-old (I/LnJ × PyMT) F_1 mammary gland; **3–5,** FVB/NJ tumors; **6–8,** (I/LnJ × PyMT) F_1 tumors.

Although results presented in this study do not prove that the *Mtmg* loci control angiogenic tumor infiltration, the difference in intratumor microvessel density would provide a potential explanation of a paradox of the (I/LnJ × PyMT) F_1 tumors. Polyoma middle-T tumors arise almost 3 weeks earlier in (I/LnJ × PyMT) F_1 mice than in the FVB/NJ parent. It might have been expected that earlier arising tumors would grow more

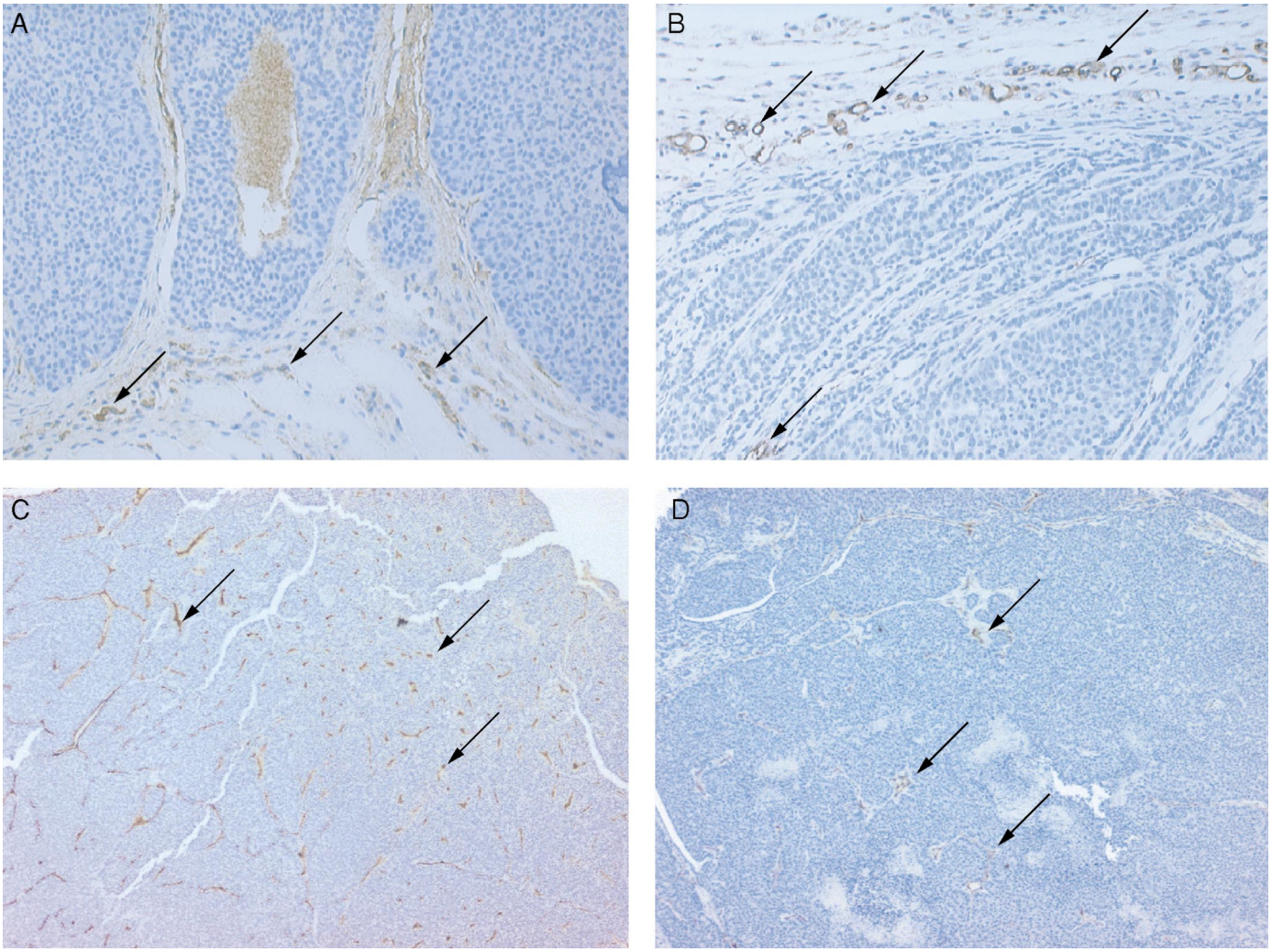

FIG. 5. Cd31 stains of FVB/NJ and I/LnJ tumors. (**A, C**) FVB/NJ tumors, showing the margin of the tumor and internal staining; (**B, D**) I/LnJ tumors. Arrows indicate select microvessels.

aggressively than tumors with longer latency. Instead, the aggressively arising tumors had a slower average growth rate than tumors of longer latency. This paradox could be explained by the pattern of microvessel recruitment. Small tumors in (I/LnJ × PyMT) F_1 animals might arise and initially grow rapidly because of the high density of microvessels around the periphery of the small tumors. The rapid growth at this stage would permit the tumors to be diagnosed early as ~2-mm masses. As the tumors continued to grow, the inability of the I/LnJ tumors to recruit intratumor microvessels could result in a decrease in proliferation capacity in the interior of the tumor. The results would be slower tumor growth rate of the (I/LnJ × PyMT) F_1 tumors compared to FVB/NJ tumors averaged over the duration of the experiment. Further analysis, however, particularly at early stages of tumor development and growth, will be required to determine the validity of this explanation. At this time, however, there is no direct link between the *Mtmg* loci and the tumor microvessel density. Microvessel density counts of tumors from all of the 126 animals of the backcross will be required to correlate the genotype/phenotype observations and confirm a direct link between the *Mtmg* loci and inhibition of tumor angiogenic infiltration.

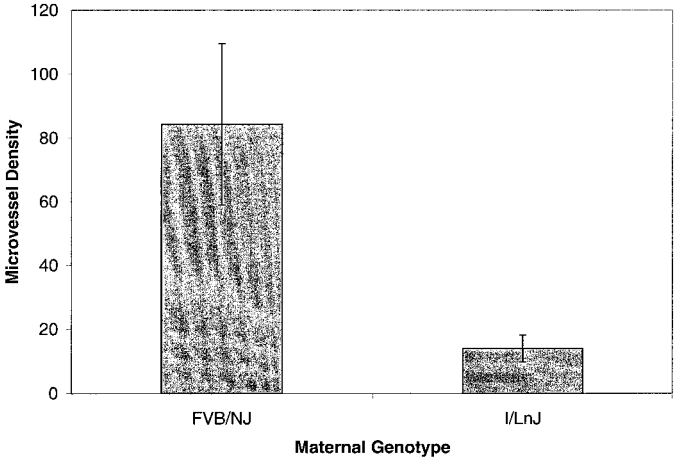

FIG. 6. Comparison of microvessel density between FVB/NJ and (I/LnJ × PyMT) F_1 tumors. The average number of Cd31-positive staining foci per 10^6 μm^2 of tumor is represented on the *y* axis.

Finally, this study has potentially important impli-

cations for clinical strategies. Antiangiogenic therapies have been of great interest and have shown great promise in laboratory and clinical trials. A number of genes have been studied as potential targets of antiangiogenic therapies. None of the genes commonly associated with angiogenesis (*Fgf2, Flt1, Flk4, Flk1, Vegf,* and *Vegfc*) are candidates for the *Mtmg* loci since they map to different chromosomes (Blake *et al.*, 2000). Angiostatin (O'Reilly *et al.*, 1994a,b) and endostatin (O'Reilly *et al.*, 1997), the naturally occurring antiangiogenesis inhibitors, are also not candidates for the genes responsible for suppression on microvessel density in this system (plasminogen, the precursor for angiostatin, maps to chromosome 17 and collagen XVIII, the precursor to endostatin is located on chromosome 10; Blake *et al.*, 2000). The results of the current study suggest that there are additional genes that are important in tumor angiogenesis. Identification and characterization of the *Mtmg* genes will hopefully provide valuable clues of their effect on tumor-vasculature interaction and potentially novel targets for clinical intervention.

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